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(54) Title: ANTIBIOTIC-METAL COMPLEXES IN THE DETECTION OF GRAM-NEGATIVE BACTERIA AND OTHER BIOLOGICAL ANALYTES

(57) Abstract: Complexes of antibi tics and metals are provided that are useful in detecting bacteria and ther bi logical analytes, and are particularly useful in detecting gram negative bacteria. The complexes are preferably chelated complexes wherein the antibi tic is a polymyxin, a colistin, an aminoglycoside, r an analog or fragment thereof. Methods f using the complexes are also provided.

ANTIBIOTIC-METAL COMPLEXES IN THE DETECTION OF GRAM-NEGATIVE BACTERIA AND OTHER BIOLOGICAL ANALYTES

TECHNICAL FIELD

The invention relates generally to detection of biological analytes, and more particularly relates to novel complexes of antibiotics and metals that useful in the catalytic detection of gram-negative bacteria and other biological analytes.

BACKGROUND ART

The term "coliform bacteria" as used herein refers to as group of bacterial genera made up of *Eschericia, Klebsiella, Enterobacter, Serratia and Citrobacter* bacteria. Coliform bacteria tend to be small, gram negative rods that may be either motile or non-motile. Coliform bacteria have complex membranes that include murein, lipoprotein, phospholipid, and lipopolysaccharide (LPS) components arranged in layers. A murein-LPS layer is about 20% of the total bacterial membrane and is responsible for bacterial cell rigidity. The LPS aids in preventing hydrophobic toxins from entering the coliform bacteria. The LPS is capable of releasing an endotoxin into a host once coliform bacteria infect the host. In human hosts, the endotoxin is released into the blood stream.

Natural competitors of coliform bacteria have evolved secondary metabolites, such as antibiotics, to overcome the LPS defense. Competitors such as soil fungi and *Streptomycetes* as well as gram positive bacteria produce antibiotics. One particular class of lipopeptide antibiotic, polymyxin, is produced by a soil microorganism, *Bacillus polymyxa.*

The polymyxins are designated by the letters A, B, C, D and E. These peptides differ in a single amino acid substitution typically being diastereomeric isomers. The polymyxins are toxic to coliform bacteria because these antibiotics bind to the LPS in the outer membrane envelope and disrupt cellular metabolism once translocated to the inner membrane cytoplasmic membrane. In particular, the polymyxins are believed to alter the structure and osmotic properties of the outer membrane. Polymyxin antibiotics are capable of binding to both animal membranes and coliform bacterial membranes.

Coliform bacteria as well as some fungi may cause urinary tract infections as well wound infections in the human host. Coliform bacteria may also cause pneumonia, meningitis, speticiemia and various gastrointestinal disorders in humans. It has been estimated that as many as 100,000 deaths in the United States each year are a consequence of gram negative infections such as coliform bacteria.

In addition to causing disease by direct infection, the endotoxin produced by coliform bacteria produces a variety of effects such as fever, fatal shock, leukocyte alterations, cytotoxicity, alterations in host response to infections, Sanarelli-Shwartzman reaction and various other undesirable metabolic changes. When coliform bacteria enter the bloodstream of a human host, endotoxic shock plays an important role in weakening the individual. About 30% of individuals with endotoxin in their blood will develop shock. About 40 to 90% of individuals in endotoxic shock die. Endotoxic shock is characterized by an inadequate supply of blood to vital organs of the host causing cellular hypoxia and metabolic failure. Survival of the host is directly proportional to the length of time needed to recognize development of bacteremia and adequate treatment of the coliform bacterial infection.

Unfortunately, to date, testing coliform bacteria, yeast and fungi has been excessively time consuming and labor intensive. While the onset of symptoms from endotoxin may be exceedingly rapid, laboratory based diagnosis will typically take days. To detect and identify coliform bacteria, it is necessary to expose suspect specimens, such as sputum, tissue, pus, body fluids and rectal swabs or feces to culture media that will allow the growth of gram negative bacteria but inhibit the growth of gram positive bacteria.

The present techniques used for this type of screening involve aseptic transfer of a sample, streaking the sample having bacterial organisms on agar plates after serial dilution and colony enumeration. This is a laborious and lengthy process requiring a period of at least 24 to 48 hours for a positive result and substantially longer for a negative result.

Additionally, test solutions containing enteric bacteria use carbohydrate and acid based indicators to demonstrate carbohydrate fermentation. Lactose and lactose analogues are the carbohydrates most frequently used in bacteria testing. This is because the majority of organisms of the genera Eschericia, Enterobacter and Klebsiella, the enteric organisms

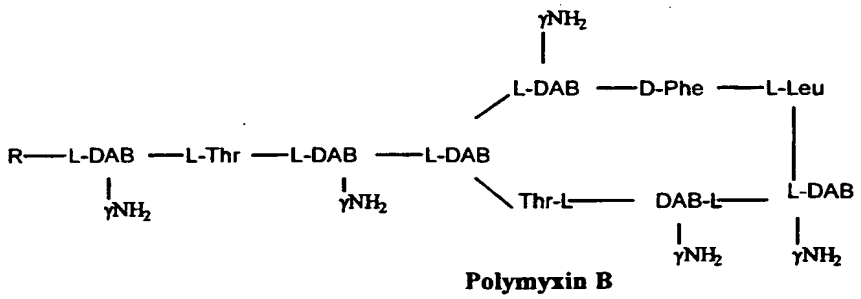
present in greatest numbers in fecal material, ferment this carbohydrate while other intestinal pathogens usually do not. Some media may also contain iron salts for the detection of hydrogen sulfide production to aid in the identification of Salmonella colonies. This approach to bacterial testing also requires a lengthy incubation time to grow enough bacteria for testing, at least 24 to 48 hours.

Other analyte tests require an organism to digest a detectable material, such as fluorescein. In other tests, an antibody, specific for an antigen on the target bacteria is labeled with fluorescein to make a fluorescent antibody. Another approach involves use of a visualization polymer coupled to a detecting agent that binds the target organism, wherein the visualization polymer is made up of detectable visualization units, such as multiple enzymes or labeled polyolefins, which are directly or indirectly bonded together (see, e.g., U.S. Patent No. 4,687,732 to Ward et al.). Another approach involves covalent conjugation of polymyxin B (PMB) and an enzyme reporter molecule, such as horseradish peroxidase (HRP), to produce a complex for use in a binding assay to detect the target organism (Applemelk et al. (1992) *Anal. Biochem.* 207:311-316). An organic "chemical tag" that comprises populations of binding agents and detectable labels has also been described (Olstein et al., U.S. Patent No. 5,750,357).

However, all of the aforementioned labeling methods suffer from the inherent steric interference introduced by the size of the tag, typically larger than 100 $Å^3$, primarily contributed by the reporter group, usually an enzyme. By contrast, the antibiotic usually being a substantially smaller molecule (20 $Å^3$) than the macro-molecular complexes described above, can readily penetrate membrane- bound receptors on the cell surface. Consequently, a continuing need exists for a sensitive and rapid method to detect extremely small amounts of target biological analytes.

DISCLOSURE OF THE INVENTION

Polymyxin B, like the other polymyxins, is a cyclic decapeptide having a high percentage of 2,4-diaminobutyric acid (also "γ,α-diaminobutyric acid" or "DAB"), a fatty acid and a mixture of D- and L-amino acids. Polymyxin B has the following structure:



Polymyxin B

DAB =γ,α diaminobutyric acid

R = Methyloctanoyl

Expected mole ratios of amino acids

DAB:Thr:Phe:Leu = 6.0:2.0:1.0:1.0

FORMULA 1

The present invention is directed to a novel antibiotic derivative that takes the form of a bound complex comprising a cyclic antibiotic and a metal. The cyclic antibiotic is preferably a polymyxin, a colistin, or an analog or fragment thereof. For example, the polymyxin or colistin can be a decapeptide--as illustrated in Formula 1, with respect to Polymyxin B--or an enzymatically derived fragment thereof, preferably a nonapeptide as described by Danner et al. (1989) *Antimicrob. Agent Chemother.* 33:1428-1434. The aforementioned nonapeptide is readily formed by removal of the 6-methyloctanoic acid moiety from Polymyxin B, for example by digestion with a standard proteolytic enzyme. As disclosed in U.S. Patent No. 5,750,357 to Olstein et al., cited *supra*, and Vaara et al. (1983) *Nature* 303:526, the cyclic nonapeptide constituent may then be easily separated from the lipid constituent and purified by a method such as liquid/liquid extraction. A preferred polymyxin or colistin fragment is one that retains an intact molecular cleft, as described below, retains bacterial binding activity and is preferably less toxic to mammalian tissues than naturally occurring polymyxins.

Preferably, although not necessarily, the metal in the complex is a transition metal or a lanthanide metal; more preferably it is copper, cobalt, iron, manganese, chromium, nickel, zinc, terbium, gadolinium, europium, or technicium. Preferably, the polymyxin is

polymyxin B (PMB). The term "polymyxin-metal complex," as used herein, is intended to encompass complexes of any polymyxin and any metal.

The polymyxin-metal complex is preferably a chelated metal complex containing one metal atom. The metal may be coordinated at four, five or six sites. Preferably, the metal binding site is within the cleft formed by the cyclized amino acids. Without wishing to be bound by theory, it is believed that this conformation may least inhibit the electrostatic interactions of the side chain amino groups of the diaminobutyric acid moieties with the anionic charged groups in lipopolysaccharide (LPS), since these interactions are crucial to LPS binding and anti-microbial activity. Any covalent modification of the side chain amino groups abolishes biological activity of the antibiotic (see Table 1, Example 4, *infra*).

The polymyxin-metal complex of the invention is unique in that it allows detection of gram negative bacteria by covalently linking the polymyxin to a detectable label. Polymyxin-metal complexes can directly catalyze peroxide-driven chemiluminescent reactions (for example, reactions involving luminol, its aromatic derivatives, lucigenin, penicillin, luciferin and other polyaromatic phthalylhydrazides) without the use of an enzyme catalyst such as horseradish peroxidase or microperoxidase. Moreover, while most organic complexes of polymyxin do not retain their anti-microbial activity (again, as shown in Table 1, Example 4, *infra*), the polymyxin-metal complexes retain substantially full anti-microbial activity.

Many of these complexes have optical properties, e.g. fluorescence, UV or visible light absorption. For example, PMB-metal complexes of terbium and europium are fluorescent and provide a visible emission in the blue band when excited with 350 nm light; PMB-metal complexes of iron and cobalt are colored and can readily be followed by spectrophotometry using techniques known to those of ordinary skill in the art and described in the pertinent texts and literature. Thus, the polymyxin-metal complex can be readily purified using standard chromatographic techniques such as gel filtration or dialysis procedures because it can be followed visually with either visible absorbance or fluorescence depending on the type of complex.

Polymyxin B has been documented to bind tightly to immobilized LPS, and this has in fact been a method for removing endotoxins from pharmaceuticals and biologicals (Issekutz (1983) *J. Immunol. Method* 61:275-281).

The invention further provides a method for detecting gram-negative bacteria comprising adding a polymyxin-metal complex to a sample suspected of containing gram-negative bacteria, washing away the unbound complex, adding a chemiluminescent agent such as luminol or lucigenin, and then measuring the resulting luminescence using a luminometer or suitably configured photo-detection device.

After synthesis, the polymyxin-metal complexes of the invention can be freeze-dried or spray dried and are preferably stored in the dark, then reconstituted in water or aqueous buffers, buffered from pH 4.5 to 7.0, for later use. Dried PMB-metal complex can be stored indefinitely with or without refrigeration, preferably stored in the dark and in the absence of molecular oxygen.

The polymyxin-metal complexes of the invention are capable of numerous and varied diagnostic and therapeutic uses. For example, gram negative bacteria can rapidly be detected in any environment, e.g., in quality control efforts in food processing and medical device sterilization. Polymyxin-metal complexes can be used to label monoclonal antibodies by cross-linking the complex to an antibody by any number of conventional cross-linking protocols. Paramagnetic complexes containing gadolinium as the chelated metal could be cross-linked to anti-tumor antibodies for use in medical magnetic imaging applications. Gadolinium, an element with a very large magnetic moment and high magnetic susceptability, is the preferred label for magnetic resonance imaging in high magnetic field strength instruments because of its superior spatial resolution in modern MRI equipment.

Therapeutic use of a polymyxin-metal Mab complex is also envisioned, either as a targeted bifunctional imaging/therapeutic agent or as a purely therapeutic agent. Preferred complexes are comprised of enzymatic fragments of polymyxin, such as the polymyxin nonapeptide, because of their lower mammalian toxicity. Complexes of polymyxin with radioactive technicium (^{99}Tc) attached to an Mab or other delivery/carrier molecule also have potential as targeted therapeutic agents. These diagnostic and therapeutic uses have great promise in the fields of cancer and AIDS treatment. Peptide-metal complexes like the

polymyxin-metal complexes of the present invention are preferred over protein-metal complexes for these uses because they are less likely to be involved with non-specific interactions, thereby reducing detection background levels.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the UV-visible spectrum of an isolated iron-polymyxin B complex at 1.5 mg/mL in phosphate buffer, pH 6.5.

Figure 2 shows the UV-visible spectrum of an isolated cobalt-polymyxin B complex at 1.5 mg/mL in phosphate buffer, pH 6.5.

Figure 3 shows the UV-visible spectrum of an isolated copper-polymyxin B complex at 1.5 mg/mL in phosphate buffer, pH 6.5.

Figure 4 shows the overlayed UV-visible spectra obtained for terbium, iron and copper polymyxin B complexes.

Figure 5 illustrates in graph form the results obtained upon chemiluminescent titration of E.coli O157:H7, signal corrected/background ratios versus serial dilutions of cell stock.

Figure 6 illustrates in graph form the results obtained upon chemiluminescent titration of Salmonella enteriditis, signal corrected/background ratios versus serial dilutions of cell stock.

MODES FOR CARRYING OUT THE INVENTION

DEFINITIONS AND OVERVIEW:

Before the present invention is described in detail, it is to be understood that unless otherwise indicated this invention is not limited to specific antibiotics, metals, ligands or the like, as such may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only and is not intended to limit the scope of the present invention.

It must be noted that as used herein and in the claims, the singular forms "a," "and" and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "an antibiotic" includes two or more antibiotics, reference to "a complex" includes two or more complexes, and so forth.

"Optional" or "optionally" means that the subsequently described circumstance may or may not occur, so that the description includes instances where the circumstance occurs and instances where it does not. For example, recitation of a chemical modification as "optionally" encompasses both the compound as chemically modified and the unmodified compound.

The present invention provides for a novel antibiotic-metal chelate constituting a new class of chemiluminescent cell labels useful for rapid detection of gram negative pathogens as well as non-pathogenic bacteria, and chemical residues of these bacteria. The invention further provides for a method of the labels' manufacture and a method for use in a rapid detection assay for bacterial pathogens.

Several references have disclosed the use of polymyxin or colistin for potential use as a diagnostic labeling reagent, such as the publications of Moore et al (Moore et al. (1986) *Antimicrob. Agent. Chemoth.* 29:496-500), Appelmelk et al (Appelmelk et al. (1992) *Anal. Biochem.* 207:311-316) and the inventor (Olstein et al., U.S. Patent No. 5,750,357, cited *supra*). The problems of antibiotic inactivation associated with chemical modification have not, however, been appreciated by these publications. Loss of antimicrobial activity upon chemical modification, in many instances, renders the modified antibiotic incapable of effectively binding to cellular receptors and therefore of limited usefulness for cellular labeling and detection.

INHERENT POLYMYXIN CHELATION:

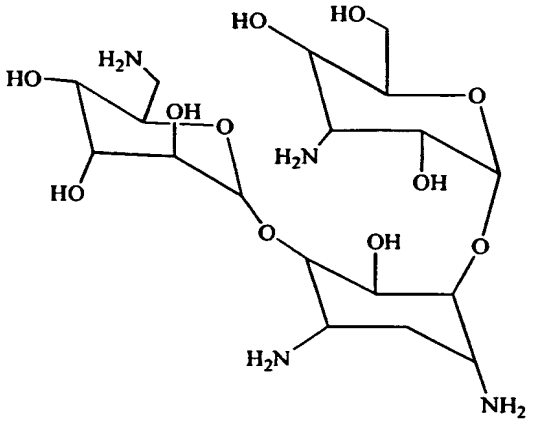
It has been observed that antibiotics of the polymyxin and colistin type tightly bind a range of metals in aqueous solution, a finding unreported in the literature. Antibiotics, particularly cationic antibiotics including polymyxins, colistins and aminoglycosides, will spontaneously chelate a range of transition metals or lanthanides in aqueous solution. The binding interaction is sufficiently tight to permit isolation of the antibiotic complex by gel filtration or dialysis, which would ordinarily separate high molecular weight compounds from simple metal salts. The strong absorbance bands exhibited by the antibiotic-metal complex permit the absorbance of the peptide chromophore at 270 nm and the visible absorbance bands at 400 nm to be used to follow purification of the complex. The efficient chelation of metals is presumably due to formation of a cleft within the structure of the

antibiotic, providing both carbonyl oxygens and amide nitrogens to contribute electron density for orbital overlap in the outer electron orbitals of a metal atom.

Optionally, addition of metal chelating ligands is possible with these antibiotics. While the polymyxins and colistin may lose appreciable anti-microbial activity, the aminoglycosides do not, allowing for efficient chemical modification of these antibiotics. A major constituent of the aminoglycosides are amino sugars, which permit several covalent modifications to be made to the amino groups on the antibiotics. The aminoglycoside kanamycin (depicted in Formula 2) is a preferred aminoglycoside, although other aminoglycosides may also be used, e.g., amikacin, streptomycin, paromomycin and gentamycin. Reductive alkylation with the aromatic carboxaldehydes, the monocarboxaldehyde of 2,2'-dipyridine, salicylaldehyde or protocatechualdehyde, for example, would produce a suitable metal binding cavity in the aminoglycoside molecule to chelate several transition metals such as copper, nickel, zinc, technetium, and preferably cobalt, iron, manganese, or chromium. The aforementioned ligands, including 2,2'-dipyridyl monocarboxlic acid, salicylic acid, and protocatechuic acid, could alternatively be grafted onto the antibiotic through an amide linkage as preformed, isolated N-hydroxysuccinimide esters. The ligands could either be used pre-loaded with the metals as reactive chelates, or optionally, chelated after the conjugates are formed.

A further embodiment of the present invention relates to the use of enzymatically or chemically derived fragments of the polymyxins and colistins in these metal complexes. Vaara et al. (1983) *Nature* 303:526 have shown that a papain digest of polymyxin yields a cyclic nonapeptide fragment, which while no longer exhibiting anti-microbial activity, still binds to the outer membrane envelope of gram negative bacteria and causes loss of membrane structure. This polymyxin nonapeptide fragment was also shown to be substantially less toxic to mammalian tissues than the native antibiotic (Danner et al., *supra*). The fragment can be used to generate metal-binding cavities in proteins and bio-molecules of interest. An example of this application is to prepare an imaging diagnostic reagent by cross-linking the nonapeptide fragment to an anti-tumor monoclonal antibody using a hetero-bifunctional reagent, such as N-hydroxysuccinimide-activated N-propionylmaleimide. The malylated peptide would then react with a native sulfhydryl on the antibody or a sulfhydryl introduced by treatment with a thiolating reagent such as

iminothiolane. Once the peptide is grafted onto the antibody sidechain(s) an atom or more, of gadolinium, a preferred metal useful for magnetic resonance imaging, could be chelated to the conjugate. Alternatively, it is possible to substitute an atom of technicium 99, a short-lived radio-isotope, into the conjugate which could then be used as a medical tracer for gamma scintillography.

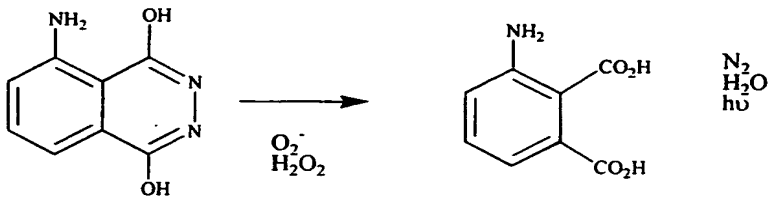


Kanamycin

FORMULA 2

METALS OF THE ANTIBIOTIC-METAL COMPLEX:

The preferred metals of the present invention include the transition metals and the lanthanides. The transition metals are particularly preferred because of their high oxidation-reduction activity in neutral aqueous media. It is likely that these metals catalyze the process of oxidizing chemiluminescent substrates, such as luminol by hydrogen peroxide, Scheme 1 (Rost et al. (1998) *J. Biolumin. Chemilumin.* 13:355-363).



SCHEME 1

The metal catalyzes the formation of superoxide and other radical oxygen species which are reactants in the chemiluminescent reaction of Scheme 1. Factors influencing the efficiency of individual metals include pH, ionic strength and oxidation state. Chelation chemistries that would alter the oxidation state or steric availability of the metals during catalysis could also influence the optimum catalytic activity as sensed by the time dependent emission of photons.

Preferred transition metals measured in the antibiotic-metal complex of the present include iron, copper, cobalt, chromium, nickel, manganese, zinc and technicium. The most preferred metals, iron, cobalt, manganese and chromium, yield the most catalytically active complexes on a molar basis.

Another preferred class of metal chelates of the present invention comprise heavy metals in the lanthanide series, including terbium, europium, gadolinium and lutitium. A unique and useful aspect of terbium and europium complexes is that neither the metal salts nor the antibiotic are fluorescent; however, some of the chelates are fluorescent. Upon addition of the lanthanide salts, terbium or europium chloride, to solutions of polymyxin, a blue fluorescent emission can be observed at 400-450 nm when illuminated with 330 nm light. The polymyxin B-terbium complex is also useful as an epifluorescence microscopy label for E.coli and Salmonella cells.

METHODS OF ANTIBIOTIC-METAL COMPLEX PREPARATION:

Preferred methods for the preparation of the antibiotic-metal complex of the present invention can be readily ascertained by those skilled in the art. Complexes are readily prepared in either water or dilute buffers, preferably volatile buffers, such as acetic acid, ammonium acetate, and ammonium bicarbonate. Crystalline or powdered antibiotic

is dissolved in aqueous medium, in concentrated solution, greater than 0.5 M, and water soluble metal salts are added to provide a slight molar excess over antibiotic. Chelates formed in solution can be isolated by dialysis in narrow-pore molecular weight cut-off tubing (e.g., as available from Spectro-Por) or by gel filtration on GPC media such as Sephadex G-25. The effluent carrying the antibiotic complex can be dried preferably by freeze drying or, alternatively, by spray drying.

Antibiotic-metal complexes isolated by the aforementioned procedures can, optionally, be further characterized by combustion analysis, NMR, and electronic spectroscopy. These procedures should also be accompanied by a bio-assay method to ensure preservation of bacterial binding activity, and/or anti-microbial activity. A useful bio-assay can be conducted using immobilized target residues, such as intact lipopolysaccharide, or lipid A, both of which are available from Sigma Chemical Co. Alternatively, an end-point determination for Minimum Inhibitory Concentration (MIC) of the antibiotic can be conducted according to standard microbiological procedures.

BINDING OF ANTIBIOTIC-METAL COMPLEX TO INTACT BACTERIA AND CHEMICAL RESIDUES:

The methods of the present invention are suitable for use in rapidly detecting gram negative pathogens in samples as diverse as drinking water, hamburger and blood. For drinking water and low protein solutions, samples require concentration with thin film type-membranes so that captured bacterial cells can be resuspended in a small volume for analysis. More concentrated samples such as biological fluids and foodstuffs lend themselves to processing with rapid isolation techniques such as immuno-magnetic micro-beads, or high density immuno-silica micro-beads.

The core utility of the antibiotic-metal complexes of the present invention is the binding activity to both intact and bacterial residues. A preferred embodiment is a simple binding assay consisting of labeling gram positive cells in suspension, pelleting the cells by centrifugation or by immuno-sedimentation, washing unbound label, and detection with chemiluminescent reagents has been demonstrated. Bacterial cells are diluted from stock cultures containing in excess of 10^6 colony forming units (CFU) per mL in peptone water. The cell suspensions are labeled at room temperature with antibiotic-metal complex of the

invention at concentrations from 0.01 to 0.05 mg/mL in peptone water for a minimum of ten minutes. The labeled cells can, optionally, be collected by centrifugation, filtration on micro-porous filters of the polycarbonate film type (Osmonics, Inc.) or rapid immuno-sedimentation using antibody-bound agarose or silica micro-beads. The labeled cells are then washed and resuspended in peptone water for assay with preferably, hydrogen peroxide/luminol or any number of oxidizable chemiluminescent, including lucigenin, penicillin and the like.

Potential bacterial targets for the antibiotic-metal complex of the present invention include E.coli, Salmonella species, Campylobacter species, all other gram negative organisms within Enterobacteriace as well as all other aerobic and anaerobic gram negative organisms.

Preferred separation methods for target pathogens include immuno-sedimentation using either magnetically accumulated micro-beads or gravity sedimentation. Several methods for isolation of pathogens from food and water have been published, e.g., Fratamico (1992) *Food Microbiol.* 9:105-113, and Pyle (1999) *Appl. Environm. Microbiol.* 65:1966-1972). Use of these immuno-sedimentation techniques provide several advantages over the aforementioned alternative selective methods such as speed, simplicity, minimization of handling, and elimination of the need for incubation equipment.

It is to be understood that while the invention has been described in conjunction with the preferred specific embodiments thereof, the foregoing description, as well as the examples which follow, are intended to illustrate and not limit the scope of the invention. Other aspects, advantages and modifications will be apparent to those skilled in the art to which the invention pertains.

EXPERIMENTAL:

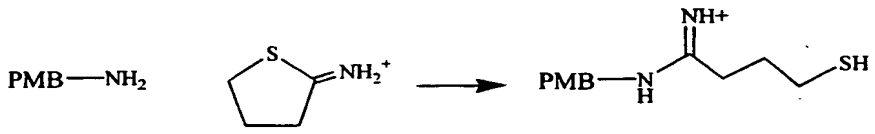
The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the antibiotic-metal complexes of the invention, and are not intended to limit the scope of what the inventors regard as their invention. Efforts have been made to ensure accuracy with respect to

numbers (e.g., amounts, temperature, etc.) but some errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, temperature is in °C and pressure is at or near atmospheric.

EXAMPLE 1

PREPARATION OF THIOLATED POLYMYXIN B

Polymyxin B was treated with one equivalent of iminothiolane, as illustrated in Scheme 2.



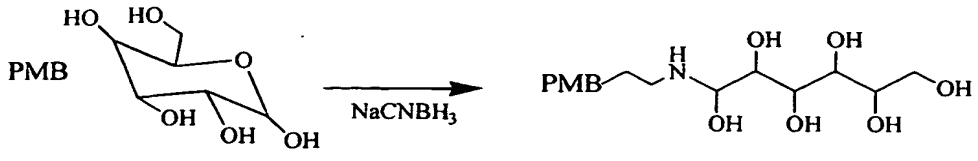
SCHEME 2

Polymyxin B (0.1 mmole, 162 mg) was dissolved in 6 mL phosphate buffer, pH 8.0, and iminothiolane (15.1 mg, 0.11 mmole, Sigma Chemical Co.) in 0.2 mL methylsulfoxide was added. The reaction mixture was incubated at room temperature for 18 hours. The thiolated antibiotic was purified by thiol exchange chromatography on thiopropyl sepharose. The sample was applied to a 2.0 x 20 column and washed with 0.1% acetic acid. The thiolated compound was eluted from the solid support by washing the column with the acetic acid containing 0.01 M mercaptoethanol. The fractions absorbing at 280 nm were pooled, desalted by flash chromatography on sephadex G-25 and freeze dried.

EXAMPLE 2

PREPARATION OF GLYCOSYLATED PMB

Polymyxin B was glycosylated by reductive alkylation by treatment of the antibiotic with one equivalent of D-mannose in the presence of sodium cyanoborohydride, as depicted in scheme 3.



Glycosylated peptide

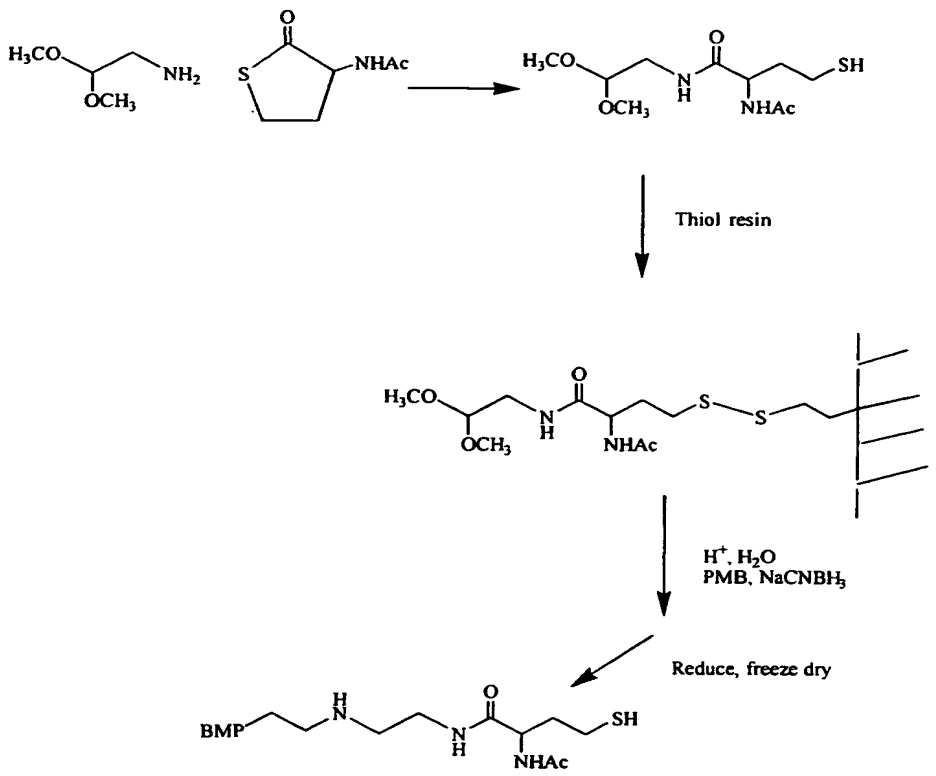
SCHEME 3

Eighty-five mg, 50 μmoles, of polymyxin B was dissolved in 4 ml of sodium phosphate buffer, pH 7.4, and ten mg mannose, 50 μmoles, was added. The reaction was stirred for fifteen minutes at room temperature and twenty mg of sodium cyanoborohydride, 300 μmoles, was added. The reaction was agitated at room temperature for eighteen hours. The product was dialyzed in 500 MWCO membrane (Spectro-Por) to remove unmodified mannose. The glycosylated antibiotic was purified on a column of aminophenylboronic acid-sepharose and freeze dried. This material was utilized for determination of anti-microbial activity.

EXAMPLE 3

ALDEHYDIC LINKAGE TO POLYMYXIN B

A derivative of polymyxin B was prepared by coupling the terminal thiol group through thiol sepharose, which has a terminal blocked aldehyde group, by reductive alkylation, Scheme 4.



SCHEME 4

Sixteen milligrams of N-acetyl-homocysteine thiolactone, 0.1 mmoles, was dissolved in 5 mL of absolute ethanol, and a six-fold molar excess, eighty milligrams, 0.6 mmoles, was added and stirred at room temperature for eighteen hours. Thiopropyl-sepharose 6B (Sigma Chemical) was re-equilibrated in a 50:50 solution of water-ethanol. A trace < 0.1 µmole of sodium borohydride was added to the coupling reaction and the product was adsorbed onto the thiopropyl-sepharose. The resin was washed with several bed volumes of water-ethanol and then distilled water. The resin was equilibrated with 4mM HCl until the pH of the effluent was < 2.0 and the material was incubated at room temperature for one hour. The polymyxin B, 0.1mmole, 162 mg antibiotic was dissolved in ten mL of 0.05 M phosphate buffer, pH 7.3. The damp thiopropyl-sepharose was suspended in the polymyxin B solution and 0.068 g of sodium cyanoborohydride was added in one mL of phosphate buffer to the suspended resin. The reaction mixture was agitated at room temperature for four hours.

The resin was then washed with several bead volumes of 0.05 M phosphate buffer until the UV absorbance @ 272 nm of the effluent was less than 0.01. The peptide derivative was eluted from the resin by washing with 0.05 M phosphate buffer containing 0.014 M β-mercaptoethanol. The sample was desalted by dialysis and freeze dried.

EXAMPLE 4

DEMONSTRATION OF LOSS OF ANTI-MICROBIAL ACTIVITY

TABLE 1

Comp.	10γ	9γ	8γ	7γ	6γ	5γ	4γ	3γ	2γ	1γ	0.13γ
PMB	-	-	-	-	-	-	-	-	-	-	+
PMB-Imthiol.	-	-	-	+	+	+	+	+	+	+	ND
Glyc-PMB	+	+	+	+	+	+	+	+	+	+	ND
ThRAl-PMB	+	+	+	+	+	+	+	+	+	+	ND

BIOASSAY FOR POLYMYXIN DERIVATIVES:

Standard Minimum Inhibitory Concentrations (MIC) were determined for the polymyxin B derivatives to determine quantitative loss of biological activity. Data were acquired using E.coli grown in liquid culture containing TSB medium.

The abbreviations in the table are as follows:

PMB= polymyxin B pentasulfate

PMB-Imthiol. = polymyxin B modified with iminothiolane (1)

Glyco-PMB = polymyxin B modified with mannose by reductive alkylation (2).

ThRAlk-PMB = polymyxin B thiolated on solid phase by reductive alkylation (3).

ND = Not done.

EXAMPLE 5

FORMATION OF THE IRON (III) COMPLEX OF POLYMYXIN B

Eighty milligrams, 0.05 mmole, of polymyxin B pentasulfate (Sigma) was dissolved in 5 mL of 0.1 M acetic acid and treated with 15 mg, 0.055 mmole, ferric chloride. After brief incubation at room temperature the preparation was gel filtered on a Sephadex G-25 column, 4.5 x10 cm. The A_{420}-absorbing material co-eluted with the UV absorbing material. These fractions were pooled and freeze dried. The freeze dried material exhibited anti-microbial activity of 2.5 μg/mL in the minimum inhibitory concentration assay. The UV-visible spectrum, Figure 1, of the isolated peptide exhibited characteristic Soret bands for planarly coordinated iron ligands such as those seen in porphyrin compounds.

EXAMPLE 6

FORMATION OF THE COBALT COMPLEX OF POLYMYXIN B

Eighty milligrams, 0.05 mmole, of polymyxin B pentasulfate was dissolved in 5 mL 0.05 M acetate buffer, pH 5.5. The antibiotic was treated with 12 mg, 0.055 mmole, cobalt chloride, and the mixture was incubated at room temperature briefly and then purified by chromatography on a Sephadex G-25 column as in example 2. The UV-absorbing fractions were collected and freeze dried. The UV-visible spectrum, Figure 2, shows a weak absorbance in the visible- near UV region centered at 340 nm.

EXAMPLE 7

FORMATION OF THE COPPER(II) POLYMYXIN B COMPLEX

Eighty milligrams of polymyxin B pentasulfate, 0.05 mmole, was dissolved in 5 mL of 0.1 M acetic acid. Twelve milligrams of $Cu(NO_3)_2$ was dissolved in 0.2 mL of buffer and added with stirring at room temperature. After ten minutes the complex was purified by chromatography on a Sephadex G-25 column. The blue copper containing band co-eluted with the U.V. -absorbing peptide fractions. The material was freeze dried and an electronic spectrum obtained, Figure 3.

EXAMPLE 8

FORMATION OF THE TERBIUM-POLYMYXIN B COMPLEX

Eighty milligrams of polymyxin B pentasulfate (Sigma), 0.05 mmole, was dissolved in 5 mL of 0.05 M acetate buffer, pH 5.5. The peptide was treated with 21 milligrams of terbium trichloride, 0.055 mmole, (Molecular Probes, Inc.). The lanthanide metal is non-fluorescent in solution; however if it is chelated, such as in the presence of EDTA or dipicolinic acid, it fluorescences with a blue emission.The complex was purified by chromatography on a Sephadex G-25 column, 2.5 x 12 cm. The complex exhibited a blue fluorescent emission, when illuminated with 330 nm light, which co-eluted with the peptide from the column. Figure 4 depicts the overlayed spectra of the metal-polymyxin complexes at comparable concentrations.

EXAMPLE 9

COMBUSTION ANALYSIS OF POLYMYXIN B- METAL COMPLEXES

Two hundred milligram samples of freeze dried samples of polymyxin B metal complexes were submitted to Galbraith Laboratories for combustion analysis. The data are summarized in Table 2.

TABLE 2

Complex	Carbon	Hydrogen	Nitrogen	Metal
PMB	40.39%	7.89%	13.60%	0%
PMB-Fe(III)	30.69%	5.35%	6.22%	1.65%
PMB-Co(II)	39.59%	6.95%	12.13%	2.65%
PMB-Mn(II)	34.85%	6.20%	9.91%	3.66%
PMB-Cu(II)	32.88%	6.03%	10.53%	4.06%

EXAMPLE 10

BINDING ACTIVITY OF IRON(II) POLYMYXIN B COMPLEX TO IMMOBILIZED LIPOPOLYSACCHARIDE

Lipopolysaccharide resin was prepared as follows. Five grams of epoxy activated sepharose 4B (Sigma) was washed with distilled water and subsequently dehydrated with

water-ethanol 20:80 (v/v), water-ethanol 50:50 (v/v) and ethanol. Ten milligrams of purified lipopolysaccharide from Salmonella Enteridis (Sigma) was dissolved in 18 mL of dimethyl sulfoxide. The dehydrated epoxy resin was suspended in the LPS solution and 0.13 mL of tributylamine was added. The suspension was agitated eighteen hours at room temperature. Unbound epoxy groups were blocked by addition of 5mL of 0.2 M glucosamine, free base, water:DMSO and incubated a further 24 hours at room temperature. The resin was progressively washed with solvents to resuspend in 100% aqueous medium.

Five mL of 2 mg/mL solution of the iron(II) polymyxin B complex in 0.05 M acetate buffer, pH 5.5 was applied to a 2mL LPS-resin column. All of the colored metal complex and the U.V. absorbing peptide was strongly bound to the resin. This data demonstrates that the complex still maintains LPS binding competency.

EXAMPLE 11

CELL TITRATION OF E.COLI O157:H7

Bacteria were diluted in sterile saline from cell concentrations of 10^8 CFU/mL to 10 CFU/mL. The cells were treated with the PMB-Co(II) complex @ 20 μg/mL for twenty minutes at room temperature. The cells were centrifuged, rinsed with 1.0 mL saline; centrifuged and re-suspended in 0.1 mL saline. Chemiluminescence was measured using 0.2mL of Luminol reagent purchased from NEN Life Sciences (Boston, MA) in a Biotrace® luminometer. Figure 5 show the titration curve for the cells, with correlation coefficient, $r^2 = 0.98$.

EXAMPLE 12

CELL TITRATION OF SALMONELLA ENTERITIS FDA STRAIN

Bacteria were diluted in sterile saline from cell concentrations of 10^8 CFU/mL to 10 CFU/mL. The cells were treated with the PMB-Co(II) complex @ 20 μg/mL for twenty minutes at room temperature. The cells were centrifuged, rinsed with 1.0 mL saline; centrifuged and re-suspended in 0.1 mL saline. Chemiluminescence was measured using 0.2mL of Luminol reagent purchased from NEN Life Sciences (Boston, MA) in a Biotrace®

luminometer. Figure 6 show the titration curve for the cells, with correlation coefficient, r^2 = 0.99

EXAMPLE 13

NEGATIVE RESPONSE FROM LISTERIA MONOCYTOGENES

Listeria monocytogenes being a gram positive organism would expect a negative response from this test since the antibiotic does not bind to gram positive cells. Bacteria were diluted in sterile saline from cell concentrations of 10^8 CFU/mL to 10 CFU/mL. The cells were treated with the PMB-Co(II) complex @ 20 μg/mL for twenty minutes at room temperature. The cells were centrifuged, rinsed with 1.0 mL saline; centrifuged and re-suspended in 0.1 mL saline. Chemiluminescence was measured using 0.2mL of Luminol reagent purchased from NEN Life Sciences (Boston, MA) in a Biotrace® luminometer. The cells exhibited background levels of chemiluminescence at all cell concentrations tested.

CLAIMS

1. A chelated complex comprised of (a) an antibiotic selected from the group consisting of polymyxins, colistins, aminoglycosides, and analogs and fragments thereof, and (b) a detectable label comprising a metal selected from the group consisting of transition metals and lanthanides, wherein the complex is capable of binding to gram negative bacterial cells or residues thereof.

2. The complex of claim 1, wherein the antibiotic is functionalized so as to contain an organic ligand capable of chelating the metal.

3. The complex of claim 2, wherein the organic ligand is bound to the antibiotic through an amide or carboxaldehyde linkage.

4. The complex of claim 2, wherein the ligand is selected from the group consisting of 2,2'-dipyridyl, phenanthrolyl, salicylyl, and catacholyl moieties.

5. The complex of claim 1, wherein the antibiotic is a polymyxin or an enzymatic or chemically derived fragment of polymyxin.

6. The complex of claim 5, wherein the polymyxin or polymyxin fragment is selected from the group consisting of polymyxin A, B, C, D and E.

7. The complex of claim 1, wherein the antibiotic is a colistin or an enzymatic or chemically derived fragment of colistin.

8. The complex of claim 7, wherein the colistin or colistin fragment is selected from the group consisting of colistin A, B, C, D and E.

9. The complex of claim 1, wherein the antibiotic is an aminoglycoside.

10. The complex of claim 9, wherein the aminoglycoside is selected from the group consisting of kanamycin, streptomycin, amikacin, gentamycin and paramomycin.

11. The complex of claim 1, wherein the metal is a transition metal.

12. The complex of claim 11, wherein the transition metal is selected from the group consisting of Cu, Co, Fe, Mn, Cr, Ni, Zn, Tc, and their isotopes.

13. The complex of claim 1, wherein the metal is a lanthanide.

14. The complex of claim 13, wherein the lanthanide is selected from the group consisting of Eu, Gd, Tb and Lu and their isotopes.

15. The complex of claim 1, wherein the complex is capable of binding to gram negative bacterial cells.

16. The complex of claim 15, wherein the bacterial cells are selected from the group consisting of Enterobacteriace, and all other classes of aerobic and anaerobic gram negative microorganisms.

17. The complex of claim 1, wherein the complex is capable of binding to residues of gram negative bacterial cells.

18. The complex of claim 17, wherein the residues are selected from the group consisting of lipopolysaccharide, lipid A and lipopolysaccharide core structures.

19. A method for synthesizing a cationic antibiotic-metal complex, comprising: (a) admixing (i) a water soluble salt of metal selected from the group consisting of transition metals and lanthanides with (ii) an antibiotic selected from the group consisting of polymyxins, colistins, aminoglycosides, and analogs and fragments thereof, in (iii) a solvent for the metal salt and the antibiotic, wherein the admixing is conducted under conditions effective to promote

chelation of the metal by the antibiotic, thereby forming a solution of the complex of the antibiotic and the metal;

(b) desalting the complex; and

(c) isolating and drying the complex.

20. The method of claim 19, wherein the solvent comprises aqueous buffer.

21. The method of claim 19, wherein step (b) comprises dialysis.

22. The method of claim 19, wherein step (b) comprises gel filtration.

23. The method of claim 19, wherein step (c) comprises freeze drying.

24. The method of claim 19, wherein step (c) comprises spray drying.

25. A diagnostic test for conducting a chemiluminescent assay of gram negative bacteria, comprising: the complex of claim 1; a source of peroxide and oxidizable substrate.

26. The diagnostic test of claim 25, wherein the oxidizable substrate is selected from the group of chemiluminescent substrates consisting of luminol and its derivatives, lucigenin, penicillin, luciferin and other polyaromatic phthalylhydrazides.

27. The diagnostic test of claim 25, wherein the source of peroxide is exogenous addition of hydrogen peroxide, or optionally benzoyl peroxide or cumyl peroxide.

28. The diagnostic test of claim 25, wherein the source of peroxide is an enzyme such as glucose or amino acid oxidase.

29. A method for conducting a chemiluminescent assay of gram negative bacteria comprising (a) isolating the target organism, (b) labeling the isolated organism with the complex of claim 1, (c) washing off unbound label and (d) detecting labeled cells by admixing a source of peroxide and an oxidizable substrate.

30. The method of claim 29, wherein step (a) comprises a sterile enrichment medium.

31. The method of claim 29, wherein step (a) comprises separation using antibody-attached micro-beads consisting of polystyrene or other synthetic latex, polymer coated ferrite or super-paramagnetic materials, silica micro-beads or cross-linked polysaccharide micro-beads e.g. agarose and sephadex.

32. The method of claim 29, wherein step (b) comprises suspension of bacteria-attached micro-beads in a solution of carrier protein or peptide and the complex of claim 1.

33. The method of claim 29, wherein step (c) comprises aspiration of the supernatant or mechanical removal of supernatant.

34. The method of claim 29, wherein step (d) comprises labeled cells and admixing peroxide with an oxidizable substrate and detection of light emission in a suitable photodetector.

35. The method of claim 34, wherein the peroxide is selected from the group consisting of hydrogen peroxide, benzoyl peroxide and cumyl peroxide.

36. The method of claim 34, wherein the oxidizable substrate is selected from the group consisting of luminol and its derivatives, lucigenin, penicillin, luciferin and other polyaromatic phthalylhydrazides.

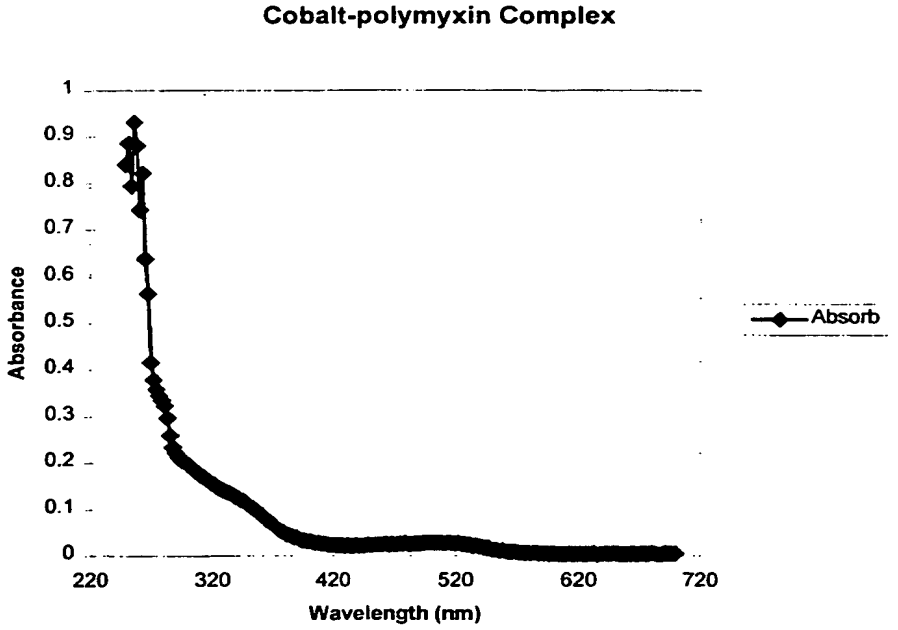


Figure 2

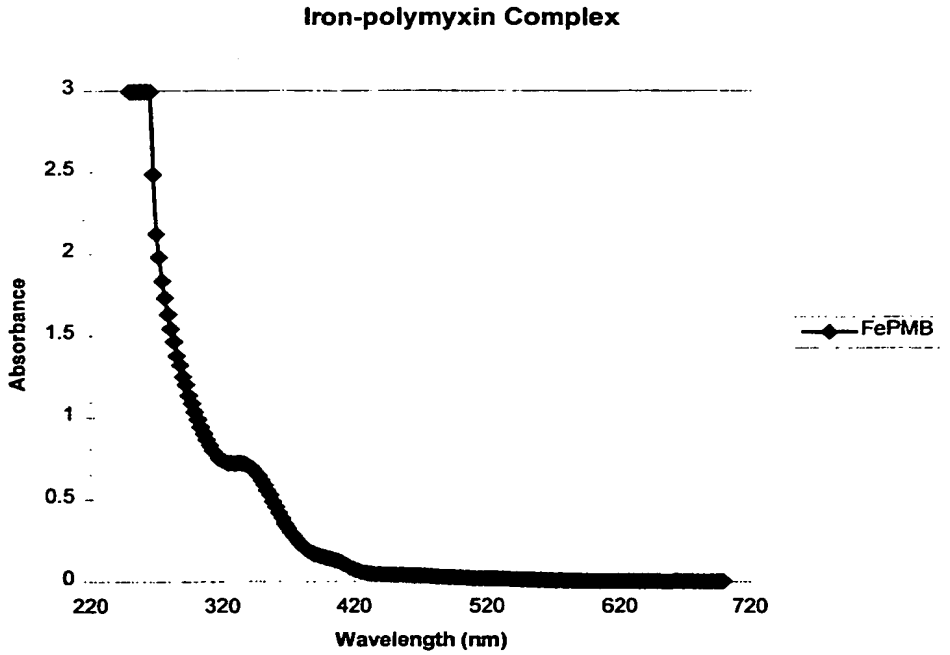


Figure 1

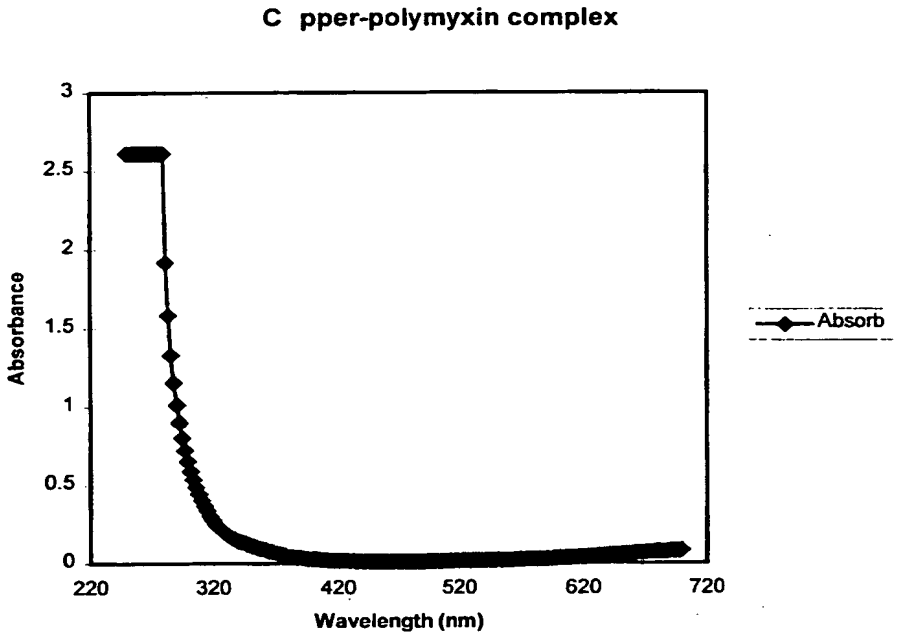


Figure 3

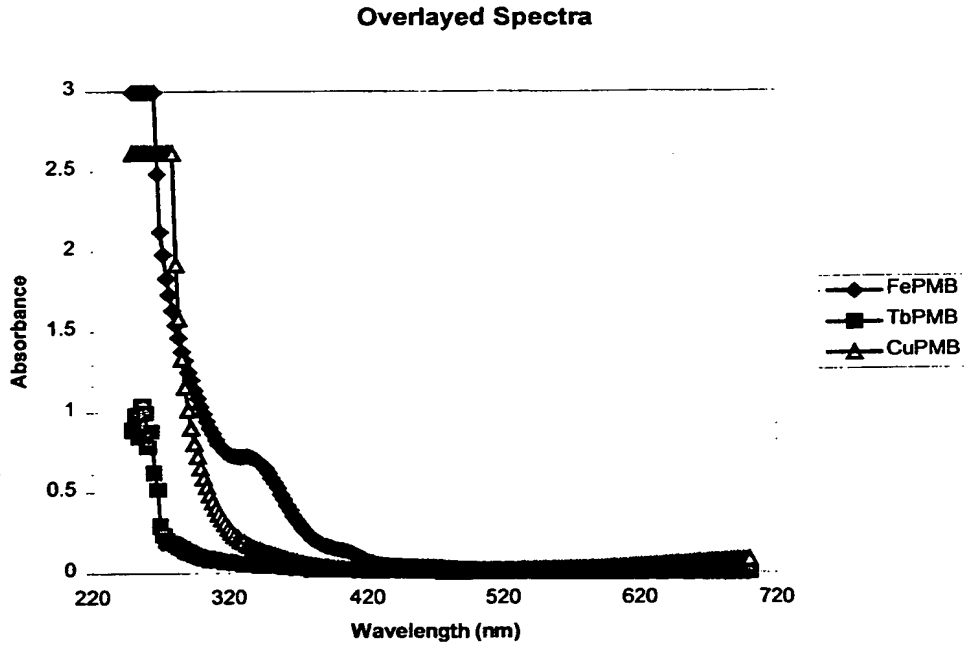


Figure 4

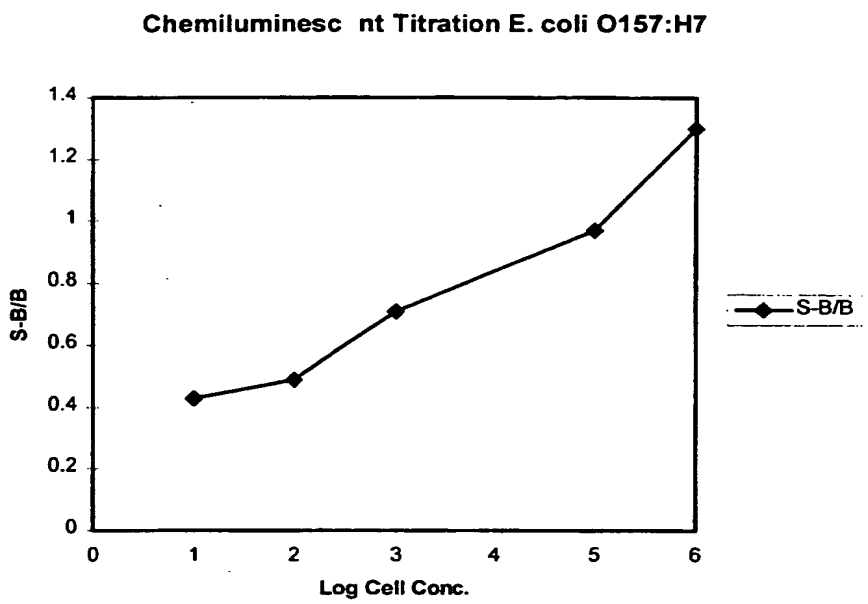


Figure 5

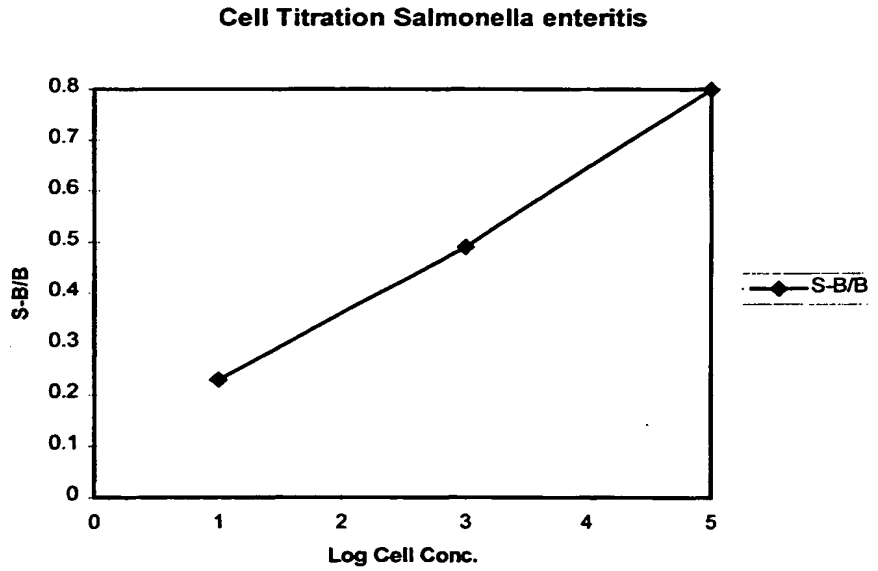


Figure 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/28577

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :G01N 33/569, 33/53

US CL :435/ 7.1, 7.2, 7.32, 7.9; 436/501, 518, 534

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/ 7.1, 7.2, 7.32, 7.9; 436/501, 518, 534

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST, DERWENT, SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,714,121 A (ALDERETE et al) 03 February 1998, see whole document.	1-36
Y	US 5,750,357 A (OLSTEIN et al.) 12 May 1998, see whole document.	1-36

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
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Date of the actual completion of the international search 16 JANUARY 2001	Date of mailing of the international search report 31 JAN 2001
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